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# Oxygen-regulated erythropoietin gene expression is dependent on a CpG methylation-free hypoxia-inducible factor-1 DNA-binding site

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The hypoxia-inducible factor-1 (HIF-1) is a transcriptional activator involved in the expression of oxygen-regulated genes such as that for erythropoietin. Following exposure to low oxygen partial pressure (hypoxia), HIF-1 binds to an hypoxia-response element located 3′ to the erythropoietin gene and confers activation of erythropoietin expression. The conserved core HIF-1 binding site (HBS) of the erythropoietin 3′ enhancer (CGTG) contains a CpG dinucleotide known to be a potential target of cytosine methylation. We found that methylation of the HBS abolishes HIF-1 DNA binding as well as hypoxic reporter gene activation, suggesting that a methylation-free HBS is mandatory for HIF-1 function. The *in vivo* methylation pattern of the erythropoietin 3′ HBS in various human cell lines and mouse organs was assessed by genomic Southern blotting using a methylation-sensitive restriction enzyme. Whereas this site was essentially methylation-free in the erythropoietin-producing cell line Hep3B, a direct correlation between erythropoietin protein expression and the degree of erythropoietin 3′ HBS methylation was found in different HepG2 sublines. However, the finding that this site is partially methylation-free in human cell lines and mouse tissues that do not express erythropoietin suggests that there might be a general selective pressure to keep this site methylation-free, independent of erythropoietin expression.

*Keywords:* erythropoietin; gene expression; hepatoma; hypoxia; methylation.

CpG methylation plays an important regulatory role in mammalian gene expression, contributing to X-chromosome inactivation, genomic imprinting as well as tissue and developmental stage-specific transcriptional regulation (reviewed in [1–4]). CpG dinucleotides are underrepresented in the mammalian genome and are often methylated if located outside of the so-called CpG islands. The human genome contains around 45 000 CpG islands of approximately 1 kb. In contrast to the bulk genome, these regions are (G+C)-rich and entirely methylation-free. CpG islands are associated with the promoters of all housekeeping genes and of 40% of the tissue-specific genes (reviewed in [5, 6]). Methylated CpG (containing 5-methylcytosine) interferes with transcription factor binding to DNA through both direct steric hindrance and the binding of repressor proteins [1]. Transcription factors that contain a CpG dinucleotide in their DNA recognition site and which are sensitive to CpG methylation include activator protein-2, nuclear factor-κB and activating transcription factor-1 (ATF-1)/cAMP-responsive element binding-1 (CREB-1) family members. In contrast, Sp1 is an example of a factor that is insensitive to CpG methylation [1]. A high number of Sp1 binding sites can be found in CpG islands where Sp1 binding activity is involved in maintaining these regions methylation-free [5].

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*Abbreviations.* AHR, aryl hydrocarbon receptor; ARNT, AHR nuclear translocator; ATF-1, activating transcription factor-1; CREB-1, cAMP-responsive element binding-1; HBS, HIF binding site; HIF, hypoxia-inducible factor.

The hypoxia-inducible factor-1 (HIF-1) is a new transcription factor containing a CpG dinucleotide in its DNA recognition site. A compilation of all currently known HIF-1 binding sites (HBSs) revealed the sequence motif RCGTGV (where R is A or G and V is A or C or G). Moreover, the first 5′ flanking nucleotide and the three 3′ flanking nucleotides are usually devoid of A residues. In 23 out of 25 cases, the core HBS is ACGTG (reviewed in [7]). HIF-1 was originally detected as a transcription factor that interacts with the hypoxia-response element of the erythropoietin gene 3′ enhancer upon exposure to low oxygen partial pressure (hypoxia, reviewed in [8, 9]). The glycoprotein hormone erythropoietin is mainly expressed in fetal liver and adult kidney, and functions as a key regulator of erythropoiesis (reviewed in [10, 11]). Subsequently, HIF-1 has also been found to convey hypoxia-inducible transcriptional activation to a number of other oxygen-regulated genes, including vascular endothelial growth factor, inducible nitric oxide synthase, heme oxygenase, transferrin and several glycolytic enzymes [7]. All of these genes are involved in maintenance of the organism's homeostasis following reductions in oxygen supply.

Biochemical purification and molecular cloning of HIF-1 revealed that it is composed of two subunits, both of which are members of the basic-helix-loop-helix-PAS family of transcription factors. While the α subunit is a novel member of this family, the β subunit is identical to the aryl hydrocarbon receptor (AHR) nuclear translocator (ARNT), previously known to heterodimerize with the AHR following binding of planar aryl hydrocarbons, such as dioxin [12]. Both HIF-1α and ARNT (HIF-1β) are ubiquitously expressed, and virtually every cell type is capable of responding to hypoxia via activation of HIF-1 [7, 8]. The gene encoding mouse HIF-1α is expressed as two different mRNA isoforms, either from a tissue-specific or a housekeeping-type CpG island-containing promoter [13, 14].

Hypoxia induces HIF-1 protein by stabilization of the HIF-1$\alpha$ subunit that is otherwise rapidly proteolytically degraded in proteasomes [15–18]. Recently, a second hypoxia-inducible factor, HIF-2$\alpha$ [7] (alternatively termed endothelial PAS domain protein-1 [19], HIF-1$\alpha$-like factor [20] or HIF-1$\alpha$-related factor [21]), has been discovered, which is mainly expressed in endothelial cells.

In this work, we aimed to examine the influence of CpG methylation on HIF-1 DNA binding and transcriptional activation. Moreover, given the ubiquitous expression of HIF-1, we analyzed to what extent CpG methylation of the HBS contributes to the tissue-specific expression of erythropoietin in human cell lines as well as in mouse tissues.

## MATERIALS AND METHODS

**Cell culture and hypoxic induction.** The human hepatoma cell lines Hep3B (HB-8064) and HepG2 (HB-8065) were obtained from the American Type Culture Collection (ATCC). The human leukemic cell line UT-7 [22] was a kind gift of C. Lacombe (Paris). The human neuroblastoma SK-N-MC (ATCC HTB-10) was kindly provided by U. Dürer (Jena). The mouse fibroblast cell line L929 (ATCC CCL-1 NCTC clone 929) was a kind gift of V. O'Donnall (Bern). The mouse hepatoma Hepa1 (also termed Hepa1c1c7) [23] was a kind gift from of L. Poellinger (Huddinge). All cells were cultured in Dulbecco's modified Eagle's medium (high glucose, Life Technologies) supplemented with 10% heat-inactivated fetal calf serum (Boehringer, Mannheim), 100 U/ml penicillin, 100 µg/ml streptomycin, 1×minimal essential medium non-essential amino acids, 2 mM L-glutamine and 1 mM sodium pyruvate (all Life Technologies) in a humidified atmosphere containing 5% $CO_2$ at 37°C. Oxygen partial pressures in the incubator (Forma Scientific, model 3319) were either 18.6 kPa (20% $O_2$ by vol., normoxia) or 0.9 kPa (1% $O_2$ by vol., hypoxia). The human epithelioid carcinoma cell line HeLaS3 (ATCC CCL-2.2), a subline of HeLa adapted to growth in suspension, was cultured and hypoxically induced as described previously [24]. Erythropoietin in the supernatant of hypoxic HepG2 cells was determined using a radioimmunoassay as described previously [25].

**Nuclear extract preparation and DNA binding assays.** Nuclear extracts were prepared and analysed by electrophoretic mobility shift assays as described previously [26]. An annealed, 5′-end-labelled oligonucleotide (5′-GCCCTACGTGCTGTCTCA-3′) derived from the human erythropoietin 3′ enhancer was used as probe [26]. Where indicated, the sense and/or antisense strand contained 5-methylcytosine at position 7 which was incorporated during synthesis of the oligonucleotides (purchased from Microsynth). For supershift analysis, the monoclonal HIF-1$\alpha$ antibody mgc3 (Camenisch, G., unpublished work) was added to the binding reaction.

**Transient transfections and reporter gene assays.** The hypoxia-responsive luciferase reporter gene construct pGLEpoHBS.3 (Fig. 3), containing a heterologous simian virus 40 promoter and three copies of the erythropoietin 3′ enhancer-derived 18 bp HIF-1-binding oligonucleotide, was described previously [26]. Hep3B and HeLaS3 cells were transiently transfected with pGLEpoHBS.3 and the parental vector pGL3Promoter (Promega) by electroporation as described [26]. Following electroporation, the cells were split and incubated for 36 h at 20% or 1% $O_2$. Luciferase activity was determined according to the manufacturer's instructions (Promega). A co-transfected $\beta$-galactosidase expression vector (pCMV$\beta$Gal) served as a reference plasmid to correct for differences in electroporation and transfection efficiency [26]. Where indicated, the plasmids were CpG methylated *in vitro* by *Sss*I methylase according to the manufacturer's instructions (New England Biolabs). Methylation efficiency was confirmed to be greater than 95% by resistance to *Tai*I restriction digestion. Alternatively, annealed and 5′ phosphorylated oligonucleotides containing the erythropoietin 3′ HBS were quantitatively ligated in a 10-fold molar excess into the dephosphorylated *Sma*I site of the parental vector pGL3Promoter. The ligation products were then directly used for transient transfections. The oligonucleotides were either in the wild-type configuration (see above), contained a mutant HBS (5′-GCCCTAAAAGCTGTCTCA-3′) [26], or were cytosine methylated on both strands (see above).

**Southern blot analysis.** Genomic DNA was isolated from various human cell lines and mouse organs by standard techniques [27] and analysed by Southern blotting as described previously [28]. To assess the CpG methylation status at the HBS in the human erythropoietin locus, genomic DNA was double digested with *Pst*I and *Tai*I according to the manufacturer's instructions (MBI Fermentas). A 0.6-kb *Pst*I fragment from the human erythropoietin cDNA 3′ end (isolated from pe49f, kindly provided by C. Shoemaker) served as hybridization probe (Fig. 4A). The mouse erythropoietin locus was analysed by simultaneous digestion of genomic DNA with *Eco*RI, *Pst*I and *Tai*I, followed by Southern hybridization to an 896-bp *Eco*RI–*Pst*I fragment (Fig. 5A) derived from a mouse genomic erythropoietin $\lambda$ phage clone isolated in our laboratory (Kreuter, R., unpublished work). Radioactive signals were recorded and quantified by phospho imaging (Molecular Dynamics), and the images were displayed using a linear relationship between signal and image intensity.

## RESULTS

**CpG methylation prevents HIF-1 DNA binding *in vitro*.** Mammalian cells are known to methylate most of the CpG dinucleotides outside of CpG islands [5]. Since the conserved core HIF-1 binding site (HBS) CGTG [7] contains such a CpG dinucleotide, we analysed the effect of CpG methylation on HIF-1 DNA binding. We first assessed whether HIF-1 is capable of binding a CpG methylated oligonucleotide derived from the human erythropoietin HBS. Oligonucleotides without (CGTG) or with a methylated HBS (mCGTG) were used to probe nuclear extracts prepared from human Hep3B and HeLaS3 cells, as well as mouse L929 and Hepa1 cells exposed to either normoxic (20% $O_2$) or hypoxic (1% $O_2$) conditions for 4 h. As shown in Fig. 1, and as we reported previously [29], a nonspecific factor and a constitutive factor bound the unmethylated erythropoietin-derived HBS probe in these nuclear extracts. Recently, we have shown that the constitutive activity reflects binding of ATF-1/CREB-1 family members to the HBS probe [26]. An additional activity of retarded electrophoretic mobility (HIF-1) appeared exclusively in hypoxic extracts. Supershift experiments using a monoclonal antibody to HIF-1$\alpha$ confirmed the identity of HIF-1 in these extracts. Interestingly, cytosine methylation within the CGTG core HBS abolished binding of HIF-1 in all hypoxic extracts tested. Moreover, binding of ATF-1/CREB-1 was also abolished whereas DNA binding activity of the nonspecific factor remained unaffected (Fig. 1). As shown in Fig. 2, hemimethylated oligonucleotide probes also did not bind to HIF-1, irrespective of whether the sense or antisense strand contained 5-methylcytosine. A theretofore unrecognized faint constitutive band became visible in this experiment which, however, did not migrate to the same position as HIF-1 or ATF-1/CREB-1.

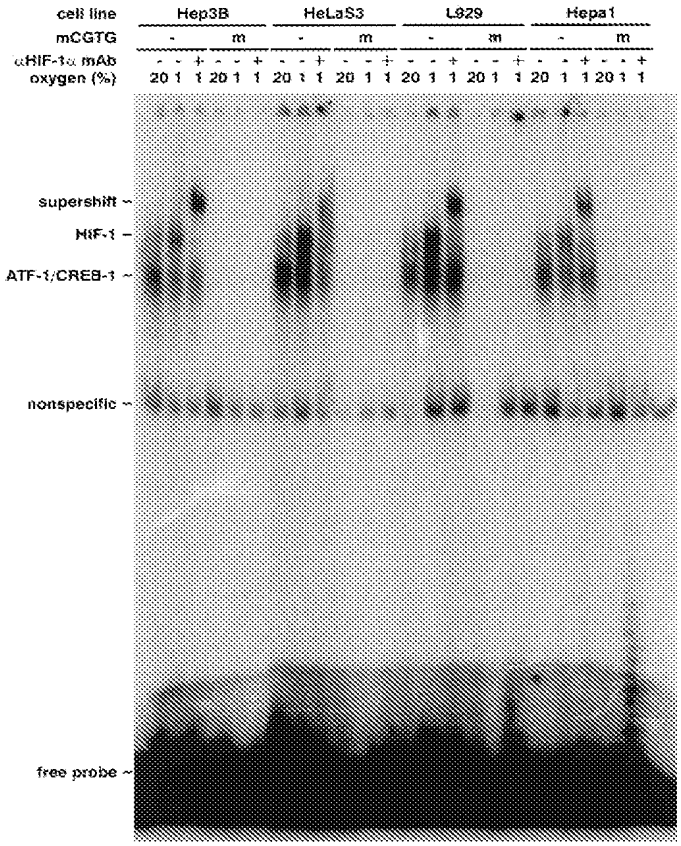


**Fig. 1. HIF-1 does not bind CpG methylated DNA.** Electrophoretic mobility shift assays of nuclear extracts were prepared from different cell lines exposed to either normoxia (20% $O_2$) or hypoxia (1% $O_2$) for 4 h. An 18-bp oligonucleotide derived from the human erythropoietin 3′ enhancer was used as probe. Where indicated, the cytosine residue at position 7 was replaced by 5-methylcytosine in both strands (mCGTG). Supershift experiments were performed using the anti HIF-1$\alpha$ monoclonal antibody mgc3.

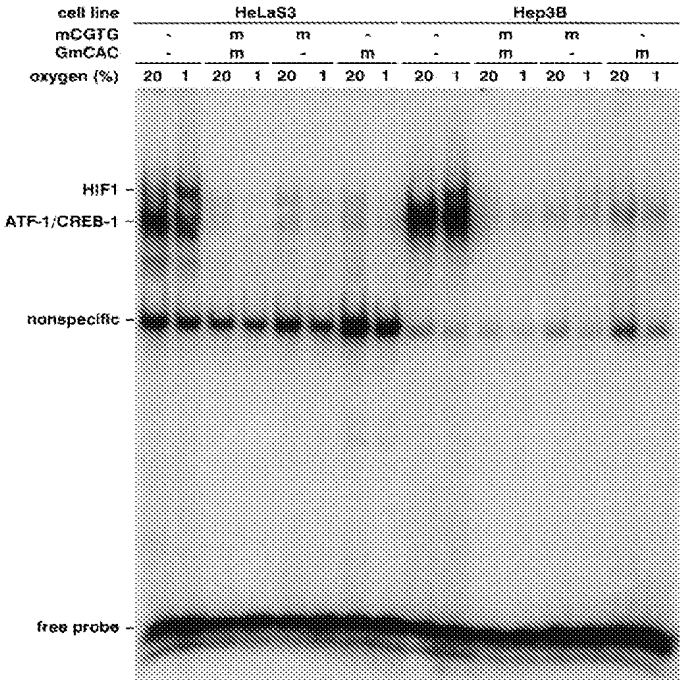


**Fig. 2. HIF-1 does not bind hemimethylated DNA.** Electrophoretic mobility shift assays were performed as described in Fig. 1. The oligonucleotide probes contained 5-methylcytosine at the sense and/or antisense strands as indicated (m).

**CpG methylation blocks transcriptional activation *in vivo*.** Impaired HIF-1 binding to a 5-methylcytosine-containing HBS *in vitro* implies that HIF-1-mediated transactivation of *in vivo* gene transcription would also be inhibited. To test this notion directly, an hypoxia-responsive reporter gene construct [23, 26], containing the firefly luciferase gene under the control of the simian virus 40 promoter and three concatamerized copies of the erythropoietin 3′ HBS (Fig. 3A), was methylated *in vitro* with the CpG-specific bacterial enzyme *Sss*I methylase [30]. Unmethylated and methylated constructs were transiently transfected into Hep3B and HeLaS3 cells, which were then split and exposed to either normoxic (20% $O_2$) or hypoxic (1% $O_2$) conditions. As shown in Fig. 3B, reporter gene expression derived from methylation-free plasmids increased under hypoxic conditions, 7.0-fold in Hep3B and 5.3-fold in HeLaS3 cells, whereas the parental vector lacking the three HBSs was only weakly hypoxia-responsive (1.3-fold in Hep3B and 1.7-fold in HeLaS3). Such an occasionally occurring weak hypoxic induction of simian virus 40 promoter-driven luciferase expression has been reported previously [26, 31, 32]. In contrast, hypoxic inducibility of CpG methylated reporter gene constructs was drastically reduced, which is about equal with the empty parental vector. Basal luciferase expression was slightly reduced by CpG methylation (approximately 5-fold in Hep3B and 2-fold in HeLaS3 cells), but this effect most probably did not account for the loss of hypoxic inducibility since luciferase expression was still clearly above background. However, to rule out the idea that CpG methylation of the promoter region or the luciferase gene itself affected hypoxic inducibility, a second series of experiments was performed where annealed, erythropoietin 3′ HBS-containing oligonucleotides were quantitatively inserted into the parental vector pGL3Promoter. As presented in Fig. 4, transient transfection of ligation products containing wild-type oligonucleotides (pGLEpoHBS.n) resulted in 3.4-fold hypoxic stimulation of luciferase expression in Hep3B and 2.7-fold in HeLaS3 cells. In contrast, transfection with ligation products containing mutant HBSs (pGLEpoHBS.n-mt) or CpG methylated HBSs (pGLEpoHBS.n-met) abolished hypoxic induction of reporter gene expression. Taken together, both DNA binding and reporter gene experiments demonstrated that a CpG methylation-free HBS is required for HIF-1 to exert its function.

**Erythropoietin expression in HepG2 cells is related to the degree of CpG methylation of the erythropoietin 3′ HBS.** Based on the results presented above, one would predict that the HBS located in the hypoxia-responsive erythropoietin 3′ enhancer should be CpG methylation-free in erythropoietin-producing cells, despite the fact that this site is not located within a CpG island. However, the question still remains whether this site is methylated in non-erythropoietin-producing cells, thereby inhibiting hypoxia-dependent erythropoietin gene expression. To answer this, we took advantage of the restriction enzyme *Tai*I that recognizes the palindromic sequence 5′-ACGT-3′ present in the erythropoietin 3′ HBS. Since this enzyme does not cleave cytosine methylated target DNA, it is ideally suited to assess the *in vivo* methylation state of the HBS. As outlined in Fig. 5A, double digestion of human genomic DNA with *Pst*I and *Tai*I results in a 707-bp fragment if the HBS is methylation-free or in an 849-bp fragment if the HBS is CpG methylated. Genomic

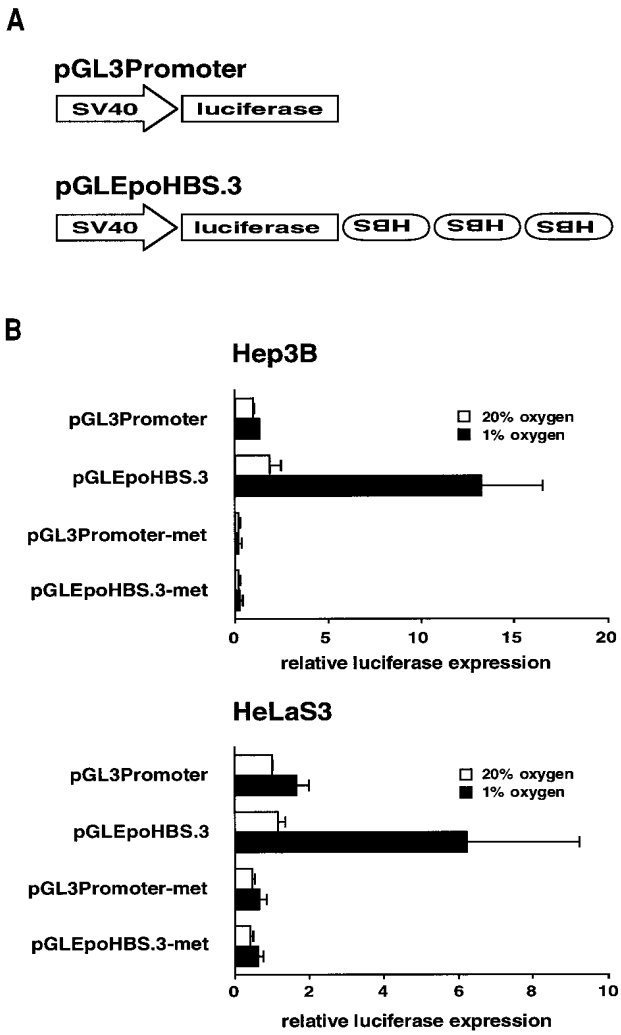


**Fig. 3. CpG methylation inhibits hypoxic reporter gene activation.** (A) Schematic representation of the reporter gene constructs used. The plasmid pGLEpoHBS.3 contained three copies of an HBS-containing oligonucleotide derived from the erythropoietin 3′ enhancer. (B) Relative luciferase reporter gene expression in Hep3B and HeLaS3 cells following transient transfection and exposure to normoxia (20% $O_2$) or hypoxia (1% $O_2$) for 36 h. Where indicated, the plasmids were *in vitro* CpG methylated (met) with *Sss*I methylase prior to transfection. A co-transfected $\beta$-galactosidase expression vector served as internal standard for transfection efficiency and extract preparation. All values were normalized to the normoxic luciferase activities obtained with the parental pGL3Promoter vector which were arbitrarily defined as 1. Means ± SD of three independent experiments are shown.

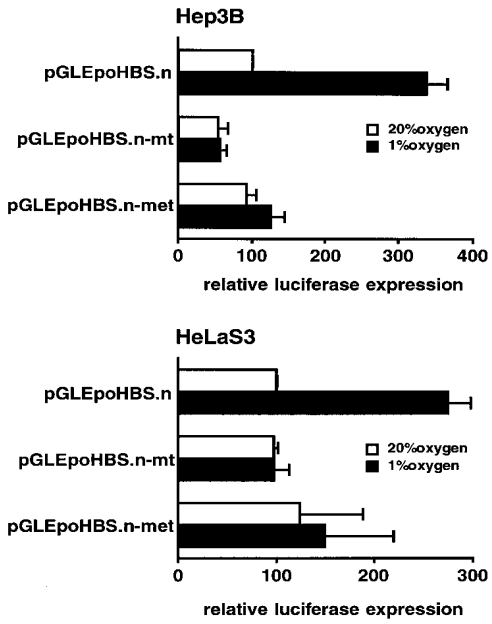


**Fig. 4. Methylation of the erythropoietin 3′ HBS abolishes hypoxic reporter gene activation.** Multiple copies of annealed oligonucleotides containing either a wild-type HBS (pGLEpoHBS.n), a mutant HBS (pGLEpoHBS.n-mt) or a CpG methylated HBS (pGLEpoHBS.n-met) were quantitatively ligated into the pGL3Promoter vector (see Fig. 3A). Following transient transfection of Hep3B and HeLaS3 cells, reporter gene activity was determined as described in Fig. 3. All values were normalized to the normoxic luciferase activities obtained with the pGLEpoHBS.n constructs which were arbitrarily defined as 1. Means ± SD of three independent experiments are shown.

DNA was isolated from the human hepatoma cell lines Hep3B and HepG2, which are the only known cell culture models capable of expressing erythropoietin in an oxygen-regulated manner [33], as well as from the randomly chosen, non-erythropoietin-producing human cell lines HeLaS3, UT-7 and SK-N-MC, and analysed by Southern blotting. As expected, the erythropoietin 3′ HBS in the erythropoietin-expressing cell line Hep3B was almost completely methylation-free (Fig. 5B). Intriguingly, this site was repeatedly found to be partially methylated (45%) in the second erythropoietin-expressing cell line HepG2. Conversely, the HBS was only partially methylated (25–50%) in all non-erythropoietin-producing control cell lines, suggesting that the mechanism keeping this site methylation-free is independent of erythropoietin expression. To determine if the methylation status of the erythropoietin 3′ HBS affects endogenous erythropoietin expression, we established several sublines of the HepG2 cell line. Three sublines (AH32, AH20 and AH39) were chosen which secreted 4.2, 7.7 and 14.2 U/l erythropoietin, respectively, following plating of an equal number of cells and incubation at 1% $O_2$ for 48 h. Interestingly, methylation of the erythropoietin 3′ HBS was 52%, 26% and 7%, respectively, indicating that the degree of methylation was inversely related to hypoxic erythropoietin production (Fig. 5C).

**Erythropoietin 3′ HBS methylation pattern in mouse tissues.** To study the contribution of CpG methylation to tissue-specific erythropoietin expression, we attempted to examine the methylation state of the 3′ HBS in erythropoietin-expressing organs of the mouse *in vivo*, using a similar strategy to that for the human cell lines (Fig. 6A). As shown in Fig. 6B, the erythropoietin-expressing tissues (liver and kidney) were less CpG methylated (both are approximately 30% methylated) than the non-erythropoietin-expressing tissues, spleen (70%), lung (40%) and tail (45%), suggesting that at least in the subfraction of hepatic and renal cells that do express erythropoietin, the 3′ HBS is CpG methylation-free. Intriguingly, despite the fact that the fetal liver is the main source of erythropoietin in the developing mouse, CpG methylation was highest in fetal liver (gestation day 13.5), decreased at gestation day 18.5, and was lowest in the adult mouse (Fig. 6C). In contrast, the degree of CpG methylation was comparable between fetal (gestation day 18.5) and adult kidney (Fig. 6C) and brain (data not shown). These findings, and the fact that a considerable fraction of cells in spleen, lung and tail were also methylation-free, suggest that methylation of

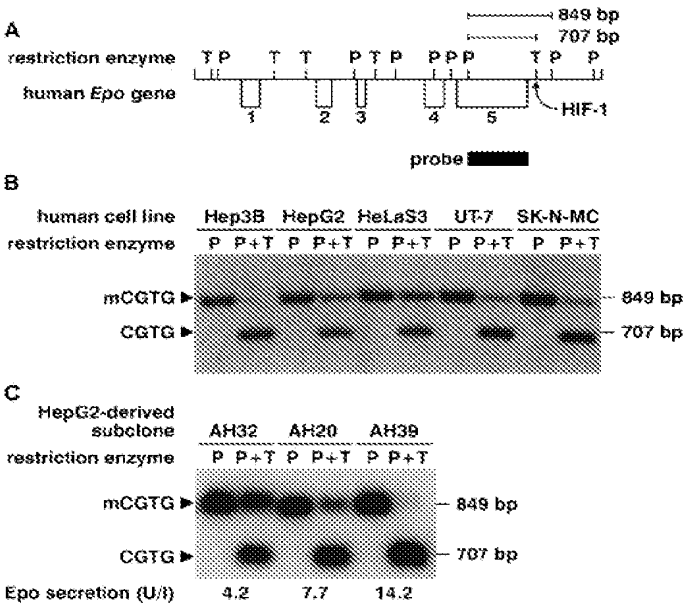


**Fig. 5. CpG methylation state of the HIF-1 binding site (HBS) in the human erythropoietin 3′ enhancer.** (A) Schematic representation of the human erythropoietin gene (*Epo*). *Pst*I (P) and *Tai*I (T) sites are indicated. (B) Southern blot analysis of genomic DNA prepared from erythropoietin-expressing (Hep3B and HepG2) and non-erythropoietin-expressing (HeLaS3, UT-7 and SK-N-MC) cell lines. (C) Southern blot analysis of HepG2-derived subclones. The respective erythropoietin (Epo) production rates after 48 h hypoxic stimulation are indicated. mCGTG, methylated HBS; CGTG, methylation-free HBS.

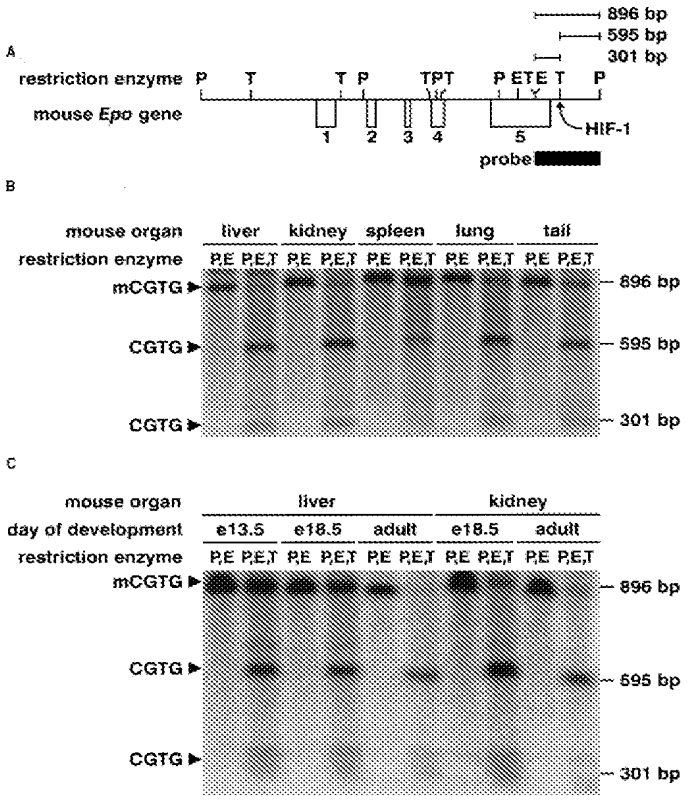


**Fig. 6. CpG methylation state of the HIF-1 binding site (HBS) in the mouse erythropoietin 3′ enhancer.** (A) Schematic representation of the mouse erythropoietin gene (*Epo*). *Eco*RI (E), *Pst*I (P) and *Tai*I (T) sites are indicated. (B) Southern blot analysis of genomic DNA prepared from erythropoietin-expressing (liver and kidney) and non-erythropoietin-expressing (spleen, lung and tail) mouse tissues. (C) Southern blot analysis of fetal (gestation days 13.5 and 18.5) and adult mouse tissues. mCGTG, methylated HBS; CGTG, methylation-free HBS.

the erythropoietin 3′ HBS does not seem to contribute to the tissue-specific and developmental-stage-specific transcriptional regulation of erythropoietin expression. Rather, as found in the cultured cell lines, there might be a general pressure to keep selectively the erythropoietin 3′ HBS CpG methylation-free, whether it is required for HIF-1 binding or not.

## DISCUSSION

In the work described in this paper, we demonstrated that the presence of 5-methylcytosine in the CpG dinucleotide of the HBS abolishes binding of HIF-1 (HIF-1$\alpha$/ARNT) as well as of the constitutive factor (ATF-1/CREB-1). These results confirmed our previous conclusion [26] that ATF-1/CREB-1 bind to the same position as HIF-1, rather than to an adjacent second putative cAMP-responsive element (the binding site of ATF-1/CREB-1) on the oligonucleotide used for the binding assays. Since the methyl group of 5-methylcytosine extends into the major groove of the DNA, our results also demonstrate that HIF-1 contacts DNA in the major groove. This is in agreement with a previous report showing, by methylation interference studies, that guanine, but not adenine methylation, also impairs HIF-1 DNA binding [34].

Analogous results to those presented here were also obtained with the dioxin-activated AHR/ARNT complex [35], which is sensitive to CpG methylation of its DNA recognition site, referred to as the xenobiotic response element TYGCGTG (Y=C or T). Thus, in the case of HIF-1, one might wonder whether the $\alpha$ or the $\beta$ subunit is methylation sensitive. The answer might be provided by two recent reports demonstrating that the AHR subunit contacts the 5′ half-site TYGC and the common ARNT heterodimerization partner binds the conserved 3′ half-site GTG [36, 37]. These data imply that HIF-1$\alpha$ also contacts the 5′ half-site AC of the HBS ACGTG. Our finding that a hemimethylated HBS abolishes HIF-1 binding when either the sense strand (corresponding to the 5′ half-site) or the antisense strand (corresponding to the 3′ half-site) contain 5-methylcytosine suggests that both the HIF-1$\alpha$ and the ARNT subunit are CpG methylation sensitive. Based on this observation, we predict also that putative heterodimers of HIF-1$\alpha$ with the recently discovered ARNT2 protein [38] most probably do not bind to a CpG methylated HBS, irrespective of whether ARNT2 will turn out to be CpG methylation sensitive or not.

The XRE element is found in multiple copies in the promoters of genes involved in xenobiotic detoxification, such as the gene encoding cytochrome *P*-450IA1. However, in contrast to the XREs of the cytochrome *P*-450IA1 gene which are located in a methylation-free CpG island [35], the erythropoietin 3′ HBS is present in a locus with average G+C content and suppressed CpG dinucleotide frequency. Thus, there must be a selective pressure to keep this site methylation-free. It is tempting to speculate that one such mechanism could be the constitutive binding of a transcription factor to the HBS. Since HIF-1 activity can only be detected under hypoxic conditions, the specific constitutive binding of ATF-1/CREB-1 to the HBS [26] might prevent methylation of the HBS. In support of this model, Hu and collaborators [39] showed, by *in vivo* footprinting, that the HBS is also occupied during normoxic growth (i.e. in the absence of

HIF-1). Consequently, since ATF-1/CREB-1 expression is ubiquitous and not restricted to erythropoietin-producing tissues, such a model would imply that non-erythropoietin-expressing cell lines and tissues are also CpG methylation-free at the erythropoietin 3′ HBS. Indeed, we found that non-erythropoietin-producing cell lines and mouse tissues contain a significant proportion of methylation-free cells.

One interesting finding of this work was that HepG2 cells were partially CpG methylated at the erythropoietin 3′ HBS, whereas Hep3B was entirely CpG methylation-free. Cultured cell lines show a tendency to inactivate genes that are not required for the cell's metabolism. This inactivation often goes along with CpG methylation of these genes [40]. Hence, the greater selective pressure to keep the erythropoietin 3′ HBS CpG methylation-free in Hep3B compared to HepG2 cells might be related to the recent report by Ohigash and co-workers [41] who presented evidence that erythropoietin might be an autocrine growth factor in Hep3B cells. In agreement with this notion, Goldberg and colleagues, who originally reported on the regulated expression of erythropoietin in Hep3B and HepG2 cell lines [33], have found that hypoxic erythropoietin induction is much greater in Hep3B cells (18-fold) than in HepG2 cells (3-fold). Our observations might also explain why HepG2 cells lose their capability to efficiently express erythropoietin after multiple rounds of passaging, an effect that forced us sporadically to re-clone this cell line in order to maintain high-level hypoxic inducibility of the erythropoietin gene (Wenger, R. H. and Gassmann, M., unpublished observations). Indeed, as shown in this work, the pattern of methylation can be clonally inherited and low levels of erythropoietin 3′ HBS methylation are inversely correlated with high hypoxic erythropoietin production rates, suggesting that methylation is an important regulator of HIF-1 activity in this cell line.

The correlation between erythropoietin expression and methylation of the 3′ HBS in mouse tissues is less conclusive. On the one hand, this might be due to the fact that only a minor fraction of liver and kidney cells express erythropoietin [42]. On the other hand, CpG methylation-free 3′ HBSs in non-erythropoietin-expressing mouse tissues might be related to our recent finding that erythropoietin is also expressed at lower levels in brain and testis in an hypoxia-inducible manner [43]. Thus, the erythropoietin 3′ HBS must be also partially functional (i.e. CpG methylation-free) at least in these organs. It is an open question whether within a given organ the subset of cells containing CpG methylation-free erythropoietin 3′ HBSs correlates with the subset of cells that potentially can express erythropoietin, and hence whether CpG methylation would contribute to the tissue-specific transcriptional regulation of erythropoietin expression. However, based on our results this hypothesis implies that, for instance, 55% of all cells in the tail are potentially capable of expressing erythropoietin in an hypoxia-inducible manner. Since this most probably is not the case, other regulatory mechanism(s), such as the interaction with tissue-specific co-activators [7,8], must also contribute to tissue-specifc erythropoietin expression. For instance, Galson and colleagues showed that hepatic nuclear factor 4, that binds to a region 20 bp downstream of the HBS in the 3′ enhancer, plays an important role in tissue-specific as well as in hypoxia-inducible erythropoietin expression [44].

So far, the location of an HBS in the 3′ flanking region of an oxygen-dependent gene is unique to erythropoietin[7]. It remains to be elucidated whether CpG methylation of 5′ HBSs present in other oxygen-dependent genes plays a significant role. However, since many of these genes (especially the glycolytic enzymes) are ubiquitously expressed and contain housekeeping-type CpG island promoters, the HBSs within these promoters are most probably methylation-free in every tissue.

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